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Hydrogen Peroxide Changes in Ischemic and Reperfused Heart

Cytochemistry and Biochemical and X-Ray Microanalysis

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Active oxygen species including hydrogen peroxide (H_2O_2) play a major role in ischemia-reperfusion injury. In the present study, changes in myocardial H_2O_2 content as well as its subcellular distribution were examined in rat hearts subjected to ischemia-reperfusion. Isolated perfused rat hearts were made globally ischemic for 20 or 30 minutes and were reperfused for different durations. H_2O_2 content in these hearts was studied biochemically and changes were correlated with the recovery of function. These hearts were also analyzed for subcellular distribution of H_2O_2. Optimal conditions of tissue processing as well as incubation medium were established for reacting cerium chloride with H_2O_2 to form cerium perhydroxide, an insoluble electron-dense product. The chemical composition of these deposits was confirmed by x-ray microanalysis. Global ischemia caused complete contractile failure in minutes and after 30 minutes of ischemia, there was a >250% increase in the myocardial H_2O_2 content. Depressed contractile function recovery in the early phase of reperfusion was accompanied by approximately a 600% increase in the myocardial H_2O_2 content. Brief pre-fixation with low concentrations of glutaraldehyde, inhibition of alkaline phosphatase, glutathione peroxidase, and catalase, post-fixation but no post-osmication, and no counterstaining yielded the best cytochemical definition of H_2O_2. In normal hearts, extremely small amounts of cerium hydroperoxide precipitates were located on the endothelial cells. X-ray microanalysis confirmed the presence of cerium in the reaction product. Ischemia resulted in a stronger reaction, particularly on the sarcolemma as well as abluminal side of the endothelial cells; and upon reperfusion, cerium precipitate reaction at these sites was more intense. In the reperfused hearts, the reaction product also appeared within mitochondria between the cristae as well as on the myofibrils, but Z-lines were devoid of any precipitate. The data support a significant increase in myocardial H_2O_2 during both the phase of ischemia and the first few minutes of reperfusion. A stronger reaction on the sarcolemma and abluminal side of endothelial cells may also indicate enhanced H_2O_2 accumulation as well as vulnerability of these sites to oxidative stress injury. (Am J Pathol 1995, 147:772–781)

A number of studies have demonstrated that the oxygen free radicals play a major role in the pathogenesis of myocardial dysfunction in a variety of conditions including ischemia-reperfusion.[1–9] Among different activated oxygen species, H_2O_2, which is not a free radical, has a relatively longer half-life and may play a significant role in oxidative stress injury.[10,11] Different oxidases of the heart, especially

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xanthine oxidase, monoamine oxidase, and NADH oxidase produce H_2O_2, which directly and/or indirectly through the production of highly reactive hydroxyl radicals can cause myocardial injury.[12–15] Superoxide anion also gives rise to H_2O_2 by spontaneous dismutation and/or catalysis by superoxide dismutase. Ultimately, H_2O_2 is reduced to water by reactions involving catalase and/or glutathione peroxidase.

To fully understand the role of free radicals in health and disease, their cellular and subcellular sources need to be understood. However, because of an extremely short half-life, *in vivo* estimation as well as distribution of active oxygen species in biological systems has been difficult. Different techniques such as electron paramagnetic resonance spectroscopy, measurement of malondialdehyde and glutathione levels, and changes in antioxidant enzymes have been used to estimate oxidative stress.[4,9,13,16–19] These approaches at best provide an aggregate assessment and do not provide any information about the *in situ* subcellular distribution of different free radicals such as can be revealed by a cytochemical approach.

In previous studies, H_2O_2 precipitation with cerium chloride ($CeCl_3$) has been used in the cytochemical demonstration of oxidases with a limited success[20,21] because of the potential contamination of the precipitate with unknown elements. Furthermore, administration of cerium by perfusion to isolated globally ischemic hearts demonstrated precipitates "almost exclusively at the luminal face of endothelial cells."[21] Such a unique luminal localization of precipitate may also have been the result of a limited access of cerium at other sites. In the same study, addition of $CeCl_3$ also caused contractile failure within seconds.[21] Thus, effects of reperfusion on function recovery in relation to H_2O_2 changes were not reported. In the present study, biochemical, cytochemical, and x-ray microanalysis approaches were used to define H_2O_2 changes during ischemia-reperfusion in rat hearts.

Materials and Methods

Ischemia-Reperfusion

For ischemia-reperfusion studies, male Wistar rats weighing 200 to 250 g were killed by decapitation, and hearts were rapidly excised and mounted on a glass cannula. Hearts were perfused retrogradely at a constant coronary perfusion rate of 8 ml/minute with a Harvard peristaltic pump. The perfusion medium consisted of a modified Krebs-Henseleit solution containing (in mmol/L) 120 NaCl, 25.4 $NaHCO_3$, 4.8 KCl, 1.2 KH_2PO_4, 0.86 $MgSO_4$, 1.25 $CaCl_2$, and 11.0 glucose and was continually gassed with a mixture of 95% O_2/5% CO_2 to maintain pH at 7.4. Both atria were trimmed away and the atrioventricular nodal tissue was crushed. These quiescent hearts were paced supramaximally at a frequency of 3 Hz during the entire experiment. Myocardial developed force was recorded by a force displacement transducer (Grass FT.03) attached by hook ligature to the apex of the heart. A small ventricular drain was made in the apex to drain the thebesian circulation. A preload tension of 2.0 g was applied to all hearts to achieve an optimal force-tension relationship. The heart was placed in a glass jacket, which was covered at the top to create a humid environment and was maintained at a constant temperature (37°C). Hearts were allowed to stabilize for 5 minutes with normally oxygenated Krebs-Henseleit buffer. Global ischemia was induced by clamping the aortic flow cannula to have zero flow rate and reperfusion was done by reinstituting the aortic flow with Krebs-Henseleit buffer. For the study of changes in developed force, 10 hearts were made globally ischemic for 30 minutes followed by reperfusion for 15 minutes.

Tissue Stabilization

Before the cytochemical reaction was started, the hearts were pre-fixed for 4 minutes by perfusion at room temperature with 0.25% glutaraldehyde in 0.1 mol/L PIPES-NaOH buffer, pH 7.8, which enabled preservation of both the ultrastructure as well as enzyme activities.[22] These partially fixed hearts were perfused for another 5 minutes with the PIPES buffer with 5 mmol/L levamisole and 10 mmol/L triazole and sliced into 40-μm-thick sections, with a TC-2 Sorvall tissue sectioner. Epicardial and endocardial layers were cut away and only the midmyocardial portions were used. For cytochemical distribution of H_2O_2, five hearts each were used at the following time points: control; 20 minutes of ischemia; 30 minutes of ischemia; and 20 minutes of ischemia followed by reperfusion for 2, 5, and 8 minutes.

Cytochemistry

The slices were incubated for 60 minutes at 37°C in the medium containing 3 mmol/L $CeCl_3$, appropriate substrates as well as inhibitors in 0.1 mol/L PIPES buffer. The list of substrates for different enzymes include 0.1 mmol/L xanthine for xanthine oxidase, 0.7 mmol/L NADH for NADH oxidase, 0.1 mmol/L

urate for urate oxidase, 0.1 mmol/L tyramine for monoamine oxidase, and 10 mmol/L D-proline for D-amino acid oxidase. The list of inhibitors of different enzymes included 10 mmol/L 3-amino-1,2,4 triazol (an inhibitor of catalase and glutathione peroxidase), 5 mmol/L levamisole (inhibitor of alkaline phosphatase), 10 mmol/L kojic acid (inhibitor of D-amino acid oxidase), 1 mmol/L KCN (inhibitor of cytochrome oxidase), 1 mmol/L chlorpromazine or 10 mmol/L chlorgylin (inhibitor of monoamine oxidase), 20 mmol/L oxonic acid (urate oxidase inhibitor), 20 mmol/L oxypurinol (xanthine oxidase inhibitor), 20 mmol/L trichloropurine (xanthine oxidase and urate oxidase inhibitor), and 1 mmol/L EDTA (5′ nucleotidase inhibitor). These chemicals were tested to optimize the production, specificity, as well as preservation of H_2O_2. The optimal incubation medium included xanthine (0.1 mmol/L), tyramine (0.1 mmol/L), levamisole (5 mmol/L), triazole (10 mmol/L), and $CeCl_3$ (3 mmol/L) in PIPES buffer. Besides using activators and inhibitors of enzymes, experiments without $CeCl_3$ and/or with catalase and glutathione peroxidase were also performed. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) and were of the highest purity grades.

Processing for Electron Microscopy

After incubation and rinsing in 0.12 mol/L cacodylate buffer (pH 7.4), sections were post-fixed in 2% osmium ferricyanide or in 2.5% glutaraldehyde in cacodylate buffer (without post-osmication) for 1.5 hours at 4°C. The sections were then dehydrated and embedded in Epon 812. Stained or unstained ultrathin sections of osmicated or nonosmicated tissues were examined. For each experimental condition, 30 micrographs were studied and the intensity of the reaction was analyzed by a person who did not know the experiment.

X-Ray Microanalysis

Semithin (100 nm), unstained, unosmicated sections were carbon coated and analyzed at a tilting angle of 30° in the scanning transmission mode of a JEOL JEM 1200 EX electron microscope equipped with a LINK 860 x-ray spectrometer. Although the probe size was 10 nm, because of electron scatter and the divergence of a convergent probe, the spatial resolution was less than 10 nm. An anticontamination device and a cold trap were used. For phosphorous analysis at 80 kV, Kα-line x-ray counts were net integrals within the window 1900 to 2140 eV. For cerium, Lα_1-line x-ray counts were net integrals within the window 4600 to 5000 eV. The detection limit under instrumental and operating conditions of analysis was between 300 and 700 ppm for both cerium and phosphorous. To precisely characterize metals in the reaction product in these sections, *in vitro* reactions were performed with defined chemicals (H_2O_2 plus $CeCl_3$ and K_3PO_4 plus $CeCl_3$). The precipitate from these *in vitro* reactions was centrifuged, embedded in Epon, sectioned in the same way, and x-ray microanalyzed.

Hydrogen Peroxide Analysis

For biochemical studies of H_2O_2, five hearts each were analyzed for control, 30 minutes of ischemia, and 30 minutes of ischemia followed by reperfusion for 2 and 8 minutes. Hearts without any pre-fixation were homogenized (10%, w/v) in a buffer containing 0.033 mol/L Na_2HPO_4 and 0.9% KCl (pH 7.4) at 4°C. The homogenate was centrifuged at 40,000 × *g* for 45 minutes and the supernatant was used for H_2O_2 analysis. Rapid peroxide-mediated oxidation of ferrous to ferric ion under acidic conditions in the presence of xylenol orange dye was followed as described.[23] For the working reagent, 1 ml of solution A containing ammonium ferrous sulfate (25 mM) and sulfuric acid (2.5 M) was mixed with 100 ml of solution B containing sorbitol (100 mM) and xylenol orange (125 μmol/L) in water. A 0.1-ml volume of supernatant was added to 1 ml of working reagent, mixed well, and incubated at room temperature for 15 minutes. The absorbance was read at 560 nm in a Spectronic 601 spectrophotometer. The values obtained from this assay were plotted against a standard curve with 1 to 50 μmol/L concentrations of H_2O_2. Peroxide standards were made fresh from a 30% H_2O_2 stock solution. The stock solution was standardized by using 43.6 $(mol/L)^{-1}$ cm^{-1} as the molar extinction coefficient at 240 nm. The absolute values of H_2O_2 are expressed in nanomoles per gram of wet weight of the heart tissue. A known amount of H_2O_2 (5 μmol/L) was added to the homogenization buffer to assess the recovery of H_2O_2 with this technique and it was found to be 87 ± 5%.

Statistical Analysis

Data are expressed as the mean ± SEM. For a statistical analysis of the data, group means were compared by one-way analysis of variance, and Bonferroni's test was used to identify differences between groups. Statistical significance was acceptable to a level of $P < 0.05$.

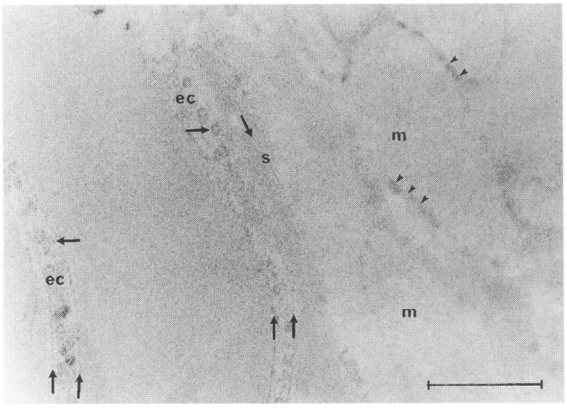


Figure 1. *Nonischemic control myocardium processed for H_2O_2 demonstration. A faint reaction (arrows) is seen on the plasma membrane as well as pinocytotic vesicles in the endothelial cells (ec). A very faint reaction (arrow) on the sarcolemma (s) is not easily discernible. Light amorphous staining around the mitochondria is nonspecific staining (arrowheads). Bar is 0.5 μm.*

Results

Distribution and Analysis of Cerium Precipitate

In pre-fixed tissue, incubated with a complete reaction mixture containing all substrates but no inhibitors, electron-dense deposits were noticed extracellularly as well as in and around all subcellular structures without any specificity of localization. These reaction products contained precipitates with varied morphological appearances including needle-like, scaly, star-shaped, and bead-like configurations. These characteristic appearances of the precipitate were distinct from an electron-dense faint staining of subcellular structures even in non-osmicated tissues. When levamisole, an inhibitor of alkaline phosphatase, was used, a significant reduction in nonspecific electron-dense deposits was noticed. Simultaneous inhibition of glutathione peroxidase as well as catalase with triazole gave reproducible definition of reaction products. Addition of the step involving 5 minutes of preperfusion of the heart with these inhibitors significantly refined the quality of the reaction (Figure 1). In all figures, tissue was non-osmicated and the sections were unstained unless mentioned otherwise. In a normal, non-ischemic tissue, an extremely fine-grain weak reaction product was seen on both luminal and abluminal sides as well as in the pinocytotic vesicles of the endothelial cells. A very faint reaction seen on the sarcolemma in the normal myocardium was not a consistent finding. Even in non-osmicated sections a light amorphous staining was noticed around mitochondria which is not to be confused with the precipitate (Figure 1). Inhibition of individual oxidases with different agents (kojic acid, KCN, chlorpromazine, oxonic acid, oxypurinol, trichloropurine, and EDTA) did not substantially modify the cytochemical distribution.

To identify the proportion of cerium perhydroxide in the precipitates in these sections, x-ray microanalysis of the precipitates obtained *in vitro* but embedded and sectioned like tissue was also done (Figure 2). The relative contributions of cerium perhydroxide and cerium phosphate (Ce/P) in precipitates were

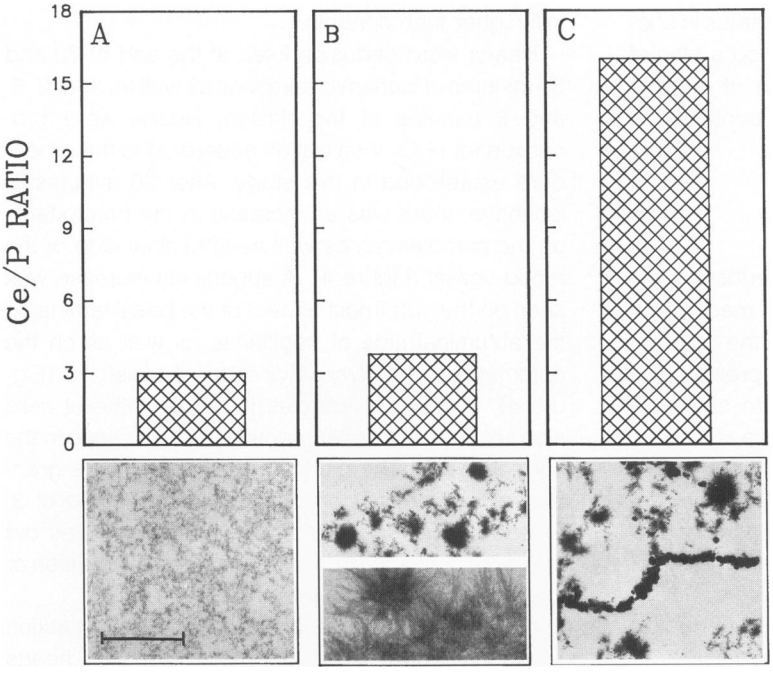


Figure 2. *X-ray microanalysis data of precipitates prepared* in vitro *is shown in the upper three panels and the corresponding characteristic morphology of each precipitate is shown in the lower three panels.* A: *Prepared from the reaction of cerium chloride with potassium phosphate.* B: *Reaction mixture as in* A: *but with a small amount of H_2O_2 also added. and* C: *Prepared from the reaction of cerium chloride with hydrogen peroxide. Magnification line shown in the lower panel is the same for all micrographs except for the lowermost micrograph in* B: *where the magnification was twice that represented by the line. Bar is 0.5 μm.*

Table 1. *Recovery of Developed Force upon Reperfusion in Isolated Rat Hearts*

Reperfusion time (minutes)	Developed force (%)
1	12 ± 5*
2	18 ± 8*
4	17 ± 8*
6	42 ± 10*
8	88 ± 10
10	90 ± 12
15	92 ± 12

Isolated perfused hearts were made ischemic for 30 minutes and reperfused for different durations. Values are expressed as a percentage of preischemic (5 minute) controls. Data are expressed as mean ± SEM of 10 hearts.

* $P < 0.01$, significantly different from preischemic control values. Control value of contractile force was 1.85 ± 0.19 g/g heart weight.

assessed from the ratios of net integrals of the cerium peaks (CeLα_1) and phosphorous peaks (PKα). Each different (Ce/P) ratio revealed a distinct morphological appearance (Figure 2, A–C). Mixing of cerium chloride and phosphate yielded the Ce/P value of 3 indicating cerium content to be three times higher than the phosphorus content. Morphologically it showed characteristic, less compact, and scaly precipitates (Figure 2A). Mixing of cerium chloride, H_2O_2, and phosphate resulted in two types of precipitates: one with a dense core surrounded by very tiny needles and the other with a star-shaped appearance (Figure 2B). The Ce/P ratio in both cases was approximately 4. A mixture of pure H_2O_2 and cerium chloride gave the highest Ce/P ratio of 16 and compact precipitates, joined end to end, took a beaded appearance (Figure 2C). Precipitates in sections from the normal heart (Fig 1) showed a ratio of approximately 3. Despite the inhibition of alkaline phosphatases, the precipitate always contained a small amount of phosphate (PKα) peak.

Ischemia-Reperfusion

The contractile force during control perfusion was normalized to 100% for each heart and mechanical function was determined by comparing the contractile activity of each heart with its own preischemic (5-minute) control value; these data are shown in Table 1. In global normothermic ischemia lasting 20 or 30 minutes, there was a complete failure of the mechanical function within minutes. Upon reperfusion, the recovery of developed force was only partial for up to 6 minutes. A significant improvement of the contractile function was noted within 7 to 9 minutes of the reperfusion and the developed force was no longer statistically different from controls.

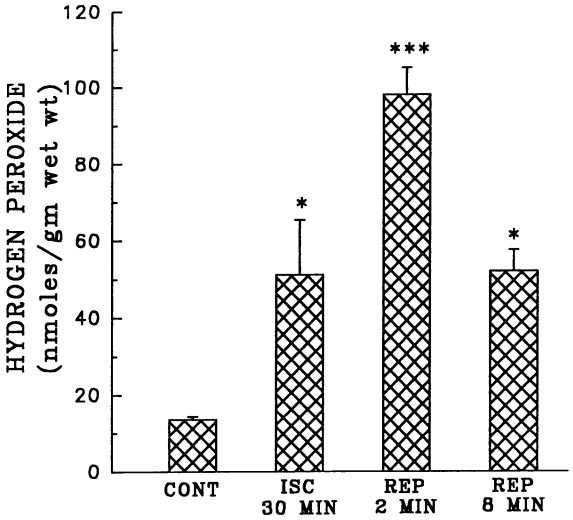


Figure 3. *Myocardial H_2O_2 in control (CONT), 30-minute ischemic (ISC), and 2- and 8-minute reperfused (REP) hearts. Although H_2O_2 recovery with this procedure was 87 ± 5%, the percent recovery in all groups as well as in the standard curve was comparable. Thus the values shown here were not corrected. Data are mean ± SE of five hearts.* ***P < 0.001 *as compared with the control group.* *P < 0.05 *compared with control and 2-minute reperfused hearts.*

Control, ischemic, and ischemic-reperfusion hearts were utilized for biochemical analysis of H_2O_2 and the data are shown in Figure 3. Global ischemia resulted in more than a 250% increase in myocardial H_2O_2. Upon reperfusion, the increase in H_2O_2 content at 2 minutes was approximately 600% as compared with control hearts. This was followed by a significant decline at 8 minutes. Although H_2O_2 levels at 8 minutes were reduced to almost one-half of that seen at 2 minutes of reperfusion, the values were still higher than controls.

Hearts were perfusion fixed at the end of 20 and 30 minutes of ischemic durations as well as after 2, 5, and 8 minutes of reperfusion. Hearts were processed for H_2O_2 localization according to the procedure established in this study. After 20 minutes of ischemia, there was an increase in the precipitates on the sarcolemma as well as abluminal side of the blood vessel (Figure 4). A substantial increase was seen on the outermost aspect of the basal lamina on the abluminal side of capillaries as well as on the outer layer of the glycocalyx of the sarcolemma (Figure 4). Pinocytotic vesicles in the endothelial cells also showed a stronger reaction than was seen in the normal hearts. The majority of the precipitate granules with a dense core showed a Ce/P ratio of 3. Extending the ischemic duration to 30 minutes did not make any significant change in the distribution or intensity of the cerium precipitate.

Intensity as well as distribution of the reaction product in 20-minute or 30-minute ischemic hearts

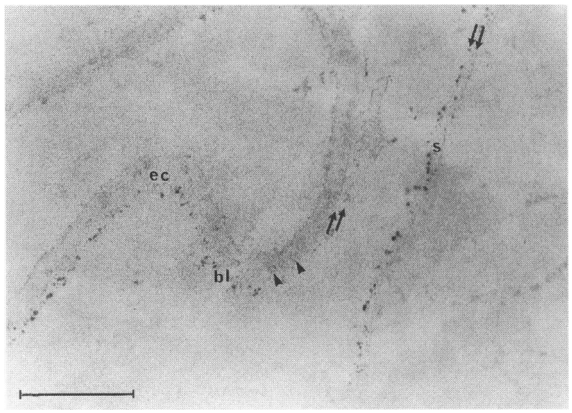


Figure 4. *Electron micrograph of a 20-minute ischemic myocardium. Processing for H_2O_2 demonstration was similar to that in Figure 1. Note a substantial increase in the precipitate mainly on the abluminal surface (bl) of the endothelial cells (ec) as well as on the outer layer of the glycocalyx of the sarcolemma (s). Two-layered localization of the precipitate can be seen (arrows). Many pinocytotic vesicles (arrowheads) also show reaction. Bar is 0.5 μm.*

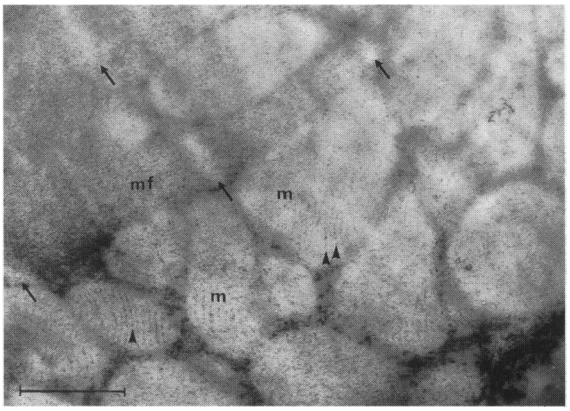


Figure 6. *High power electron micrograph of a myocyte from reperfused heart demonstrating scaly or fine grain precipitate localized on myofibrils (mf) and in between the mitochondrial cristae (m) (arrowheads). Z-lines (arrows) are free of any precipitate. Bar is 0.5 μm.*

reperfused for 2 or 5 minutes were similar and were significantly more than ischemia with no reperfusion or 8-minute reperfused hearts. Subsequent cytochemical data on H_2O_2 localization is from 20-minute ischemic hearts reperfused for 5 minutes. The precipitate was found in larger quantities on the sarcolemma as well as some other subcellular structures also showed deposits (Figures 5 to 8). Sarcolemmal localization appeared in two layers (Figure 5). At a higher magnification, an inner layer consisting of tiny granules was localized on the plasma membrane and an outer layer consisting of coarser granules or star-shaped deposits appeared on the outermost aspect of the glycocalyx (Figure 5, inset). Precipitate composed of either scaly or fine granules of relatively uniform size was also found on the myofibrils, but Z-lines were characteristically negative (Figure 6). A similar type of precipitate was also seen within the mitochondria, localized in between the cristae membranes (Figure 6). A precipitate in endothelial cells was randomly distributed throughout the cell with larger amounts confined to luminal and abluminal surfaces (Figure 7). On the abluminal surface, the precipitate appeared in two prominent layers representing plasma membrane and the outermost aspect of the basal lamina (Figures 7 and 8). Some of the pinocytotic vesicles were also intensely stained. Morphologically, there were two types of precipitates, one with a dense core (Figure 7) and the other with a star-shaped appearance (Figure 8) similar to the ones described in Figure 2B. There were also a few star-shaped precipitates present in the extracellular space not corresponding to any particular structural morphology (Figure 8).

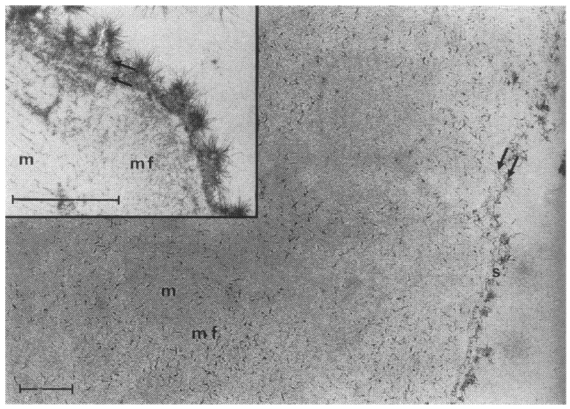


Figure 5. *H_2O_2 localization in myocardium reperfused for 5 minutes. Precipitate deposits are localized on the sarcolemma (s) in two distinct layers (arrows). Inset shows better details of star-shaped as well as fine grain precipitate on the cellular structures. Small amount of fine grain (inset) as well as scaly precipitate is restricted to myofibrils (mf) and mitochondria (m). Bar is 0.5 μm.*

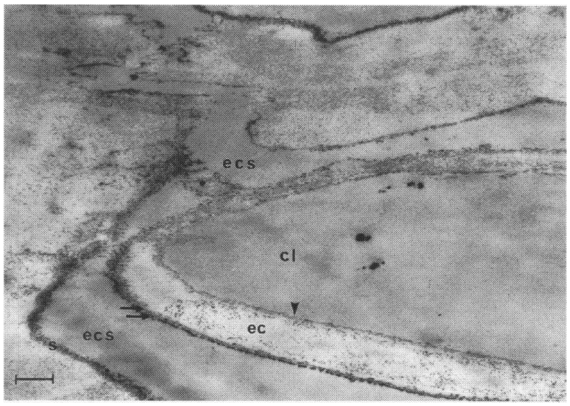


Figure 7. *Reperfused myocardium (5 minutes). Distribution of precipitate on the endothelial cell (ec). A significant increase in the reaction for H_2O_2 on surfaces facing extracellular space (ecs). Note the double-layered pattern on abluminal surface of endothelial cell (arrows) and a lower intensity of the precipitate on luminal side (arrowhead). cl, capillary lumen; s, sarcolemma. Bar is 0.5 μm.*

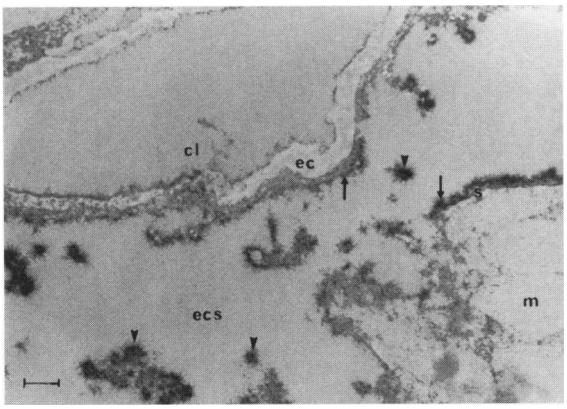


Figure 8. *Electron micrograph of a reperfused myocardium (5 minutes) demonstrating mostly spiky precipitates. An intense reaction (arrows), as a double layer on the abluminal surface of the endothelial cell (ec) and on the sarcolemma (s) can be seen. A few heavy deposits (arrowheads) are also scattered in the interstitial space (ecs), cl, capillary lumen, m, mitochondria. Bar is 0.5 μm.*

Inclusion of catalase and glutathione peroxidase in the incubation mixture prevented the formation of precipitates in the mitochondria as well as on the myofibrils and reduced the deposits on the sarcolemma and endothelial cells (data not shown). Although osmication of tissue after incubation improved contrast, a fine definition of precipitates described above was completely masked and became indistinguishable (Figure 9). The electron-dense contrast seen in osmicated tissue around mitochondria is not related to the H_2O_2 generation.[14]

Characteristic profiles of the precipitate on seven different specific sites, including sarcolemma, glycocalyx, myofibrils, mitochondria, endothelial cells, and extracellular space in non-osmicated semithin sections, were analyzed with the x-ray technique and the results are shown in Figure 10. For this analysis, 30

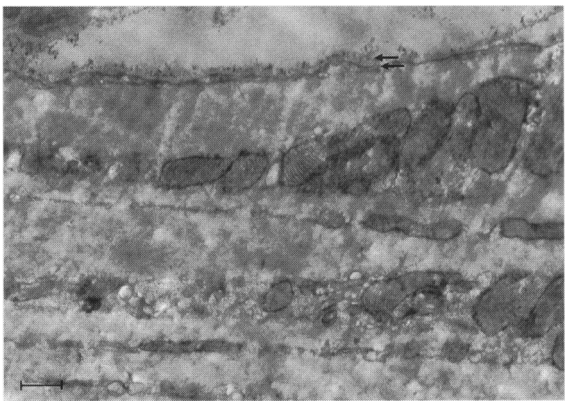
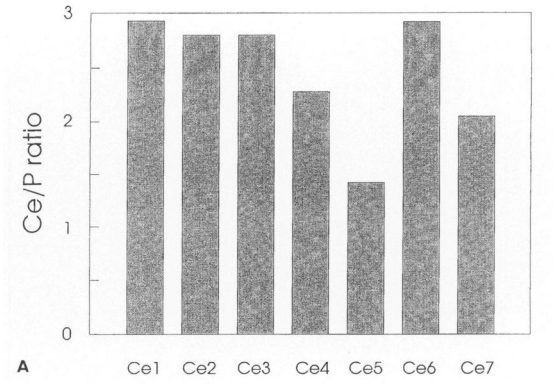


Figure 9. *In a 5-minute reperfused heart, osmication of the partially fixed tissue increased contrast. However, a fine granular precipitate in the mitochondria and on myofibrils is completely masked by the osmium staining and is no longer distinguishable. A specific precipitate on the sarcolemmal glycocalyx in a two-layered pattern can still be seen (arrows). Bar is 0.5 μm.*



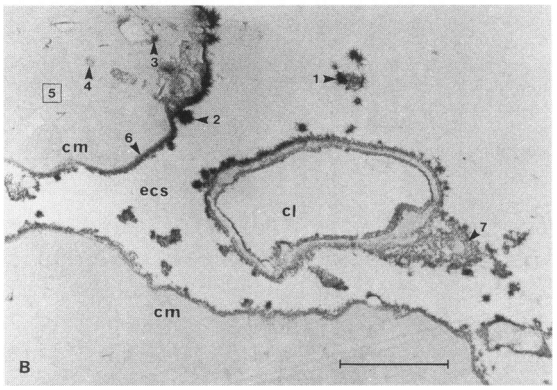


Figure 10. *X-ray microanalysis of cerium and phosphorus. The Ce/P ratio (A) as well as corresponding areas, numbered from 1 to 7, analyzed in the reperfused heart are shown (B). A higher Ce/P ratio indicates a higher content of cerium perhydroxide in the precipitate. cl, capillary lumen; cm, cardiac myocyte; ecs, extracellular space. Bar is 3.5 μm. Areas 1 to 7 were randomly selected from the structural as well as amorphous details both in the intracellular and extracellular regions.*

grids from five hearts were studied. All precipitates of cerium perhydroxide contained not only cerium but also phosphate. Myofibrils showed the weakest reaction with a Ce/P ratio of approximately 1, whereas at other sites, the reaction ratio ranged between 2 and 3. None of the sites showed the deposit ratio of 16:1, the maximal concentration of cerium perhydroxide seen in *in vitro* studies.

Discussion

The present study addresses a very important point that H_2O_2-producing sites in the myocardium are enhanced/activated during ischemia and reperfusion and can be made to express under *in vitro* conditions. Although precise sites of H_2O_2 production remain to be established, its accumulation on the sarcolemma as well as on the abluminal side of the coronary capillaries is also documented in this study. Increased activity at these yet undefined sites may explain the significant increase in the myocar-

dial content seen in this study both during ischemia and early reperfusion.

A number of histochemical and immunocytochemical methods used for the localization of oxidases in fact rely on the localization of H_2O_2 produced with a divalent reduction of O_2 ($2H^+ + O_2$ oxidases H_2O_2). Cerium ions are used to precipitate with H_2O_2 ($H_2O_2 + CeCl_3 \rightarrow Ce(OH)_2OOH$) and the insoluble reaction product lends itself to ultrastructural cytochemistry.[20] However, it has been reported that cerium avidly reacts with phosphate and the reaction has also been used for the demonstration of phosphatases.[24] Thus in previous studies, demonstration of NADH oxidase with cerium chloride was compounded by phosphatases.[14,25] In these studies, the use of levamisole for the inhibition of alkaline phosphatase as well as cyanide as NADH-cytochrome reductase inhibitor, was also recommended to avoid a false reaction. Although inhibition of alkaline phosphatase in our study clearly improved the definition of the deposits, the presence of phosphate in the precipitate was confirmed by x-ray microanalysis. However, for a precise localization of H_2O_2, based on cerium product, several factors were combined in the present study: (1) optimal conditions for tissue processing were established; (2) alkaline phosphatase was inhibited to minimize interference by phosphorus; (3) glutathione peroxidase and catalase were inhibited to conserve H_2O_2 produced; and (4) x-ray analysis was done to identify composition of the precipitate.

The use of fresh unfixed tissue always resulted in poor structure and in the diffusion of precipitates. Unlike peroxidases and catalase, oxidases (except cytochrome oxidase) are relatively sensitive to aldehyde fixation,[26,27] but the approach of a brief tissue pre-fixation proved satisfactory both for the production and conservation of H_2O_2 in the heart slices as well as for preservation of the ultrastructure. To improve details as well as intensity of the reaction product, inhibition of enzymes, namely, alkaline phosphatases, glutathione peroxidase, and catalase, was found to be essential. Addition of inhibitors of different oxidases to differentiate specific oxidases was found to be unnecessary as long as their substrates were not provided in the incubation medium. That the final reaction product involved H_2O_2 was unequivocally established by the fact that the addition of catalase and glutathione peroxidase in the medium caused the disappearance of the precipitate from intracellular sites as well as a significant reduction of the reaction on the plasma membrane of the myocytes and endothelial cells. As catalase as well as glutathione peroxidase cause a divalent reduction of H_2O_2 to water, loss of the formation of cerium perhydroxide precipitate on specific structures documents the reliability of the method for H_2O_2 localization. Thus in the present study, the optimal incubation medium included xanthine, tyramine, levamisole, triazole, and $CeCl_3$ in PIPES buffer. Brief prefixation with glutaraldehyde followed by perfusion for 5 minutes with PIPES buffer (pH 7.8) containing levamisole and triazole was found to be adequate. Post-osmication as well as staining are not recommended.

Our x-ray microanalysis data showed three times as high a reaction on the sarcolemma as well as both surfaces of endothelial cells as compared with the contractile fibers. This probably represents places of highest concentration and/or generation of H_2O_2 and may suggest that these areas are most vulnerable to oxidative stress injury. Generally, xanthine oxidase is suggested to be low in a normal heart but during reperfusion this activity increases.[12,28–30] Relatively large amounts of reaction product throughout the endothelial cells found in our experiments might support higher activity of xanthine oxidase in the reperfused rat myocardium. Another probable source of H_2O_2 could be the mitochondria. These organelles in the reperfused myocardium are damaged and may therefore make more H_2O_2 than mitochondria in control hearts. H_2O_2 localization within the mitochondria of the reperfused hearts was quite apparent in this study. The lack of any significant H_2O_2 localization in the mitochondria in control heart may suggest adequacy of H_2O_2 reduction in these organelles in normal conditions.

An increase in H_2O_2 production after reperfusion has been described by several authors[14,21,31–34] and has also been shown by the biochemical data in the present study. Cytochemical data suggest enhancement/activation of H_2O_2-producing sites as a result of ischemia and reperfusion that could be demonstrated in *in vitro* conditions in a partially fixed tissue. In addition, our study documents specific subcellular sites of H_2O_2 accumulation. Whether these were also the sites of H_2O_2 production is not defined by the data. Another interesting as well as important revelation in the present study is the significant increase in myocardial H_2O_2 caused by the ischemia alone. An increase in free radicals in coronary sinus blood within 1 to 5 minutes of coronary occlusion in dogs has been reported earlier.[35] Occurrence of free radical reactions at very low oxygen tensions has also been demonstrated in *in vitro* radiobiological studies.[36] These data support the argument that, even under reduced oxygen tension

during ischemia, H_2O_2 production as well as injury can occur.

Our finding of predominant abluminal localization contrasts with perfusion studies reporting the highest concentration of H_2O_2 and superoxide on the luminal surface of endothelial cells.[21,32] Localization of H_2O_2 and other active oxygen species "exclusively on the luminal side" in isolated hearts perfused with the reaction medium[21] may be the result of availability of the substrates as well as cerium being restricted to the luminal side. In our previous experiments of perfusion of hearts with incubation medium containing $CeCl_3$, we also found a similar localization of large electron-dense deposits on luminal sites of endothelial cells (unpublished data). Incubation of tissue slices as used in the present study eliminates penetration/diffusion problems as well as phosphate contamination engendered in the perfusion methods. This fact may also explain previous reported localization of the precipitate predominantly in the lumen as well as on the luminal surface of capillary endothelial cells.[21,32]

The increased amount of cytochemically demonstrable H_2O_2 in the early reperfusion period (2 to 5 minutes) corresponded with the biochemical data inasmuch as, at 2 minutes of reperfusion, the increase in H_2O_2 was significantly more than the control, ischemic, and ischemic-reperfused hearts at 8 minutes. These changes also correlated with the depressed contractile function. Transient post-ischemic injury, also called stunning, has been suggested to be associated with a variety of subcellular abnormalities resulting from a pathogenic action of oxygen free radicals.[37] With electron spin resonance technology, an increase in oxygen free radicals during the early reperfusion period in open chest dogs has been shown.[4] Species of oxygen radicals produced during various phases of ischemia-reperfusion remains to be defined. The present study documents the accumulation of H_2O_2 during ischemia-reperfusion. In this regard, perfusion of rat hearts with H_2O_2 has been reported to increase other active oxygen species as well as cause myocardial dysfunction.[38] An increase in H_2O_2, as one of the active oxygen species in the oxygen radical chain reaction, may not only be caused by its increased production but may also be aided by a decrease in catalase and glutathione peroxidase activities subsequent to an increase in H_2O_2. In fact, inhibition of antioxidants during hypoxia-reoxygenation as well as in ischemia-reperfusion has been documented.[10,39]

The present study partially answers the question concerning the sites of H_2O_2 accumulation and/or production. There is no definite explanation for extracellular, mitochondrial, and myofibrillar localization of H_2O_2. Although the production of H_2O_2 within mitochondria as well as at the sarcolemmal and endothelial cell membranes may be supported by oxidases, myofibrillar localization may suggest other redox reactions and/or spontaneous dismutation of the superoxide radical at these sites. Considering a relatively longer half-life of H_2O_2, the possibility of its migration to myofibrils from other sources within the cell cannot be excluded. In any case, the present study clearly shows an increase in H_2O_2 during the ischemic phase and a much larger increase within minutes of reperfusion. By using a more reliable cytochemical approach, the accumulation of H_2O_2 in well defined extracellular as well as intracellular sites is reported.

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